Nesterenkonia halotolerans sp. nov. and *Nesterenkonia xinjiangensis* sp. nov., actinobacteria from saline soils in the west of China

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The taxonomic position of two Gram-positive strains, YIM 70084^T and YIM 70097^T, isolated from hypersaline soils was determined by a polyphasic approach. Cells of strain YIM 70084^T are motile cocci, whereas those of strain YIM 70097^T are non-motile rods. The G+C contents of their DNA are 64·4 and 66·7 mol%. Both strains had chemotaxonomic markers typical of the genus *Nesterenkonia* and formed a coherent cluster with *Nesterenkonia* species in a phylogenetic inference based on 16S rDNA sequence analysis, exhibiting less than 97 % similarity to each other and to the other two type strains of the genus. Phylogenetic distinction and differences in the peptidoglycan type, composition of cell-wall sugars, phospholipid patterns, the major menaquinones and other phenotypic characteristics indicate that the strains under study represent two novel species, *Nesterenkonia halotolerans* sp. nov. (type strain YIM 70084^T = CCTCC AA 001022^T = DSM 15474^T) and *Nesterenkonia xinjiangensis* sp. nov. (type strain YIM 70097^T = CCTCC AA 001025^T = DSM 15475^T).

A phylogenetic and chemotaxonomic re-analysis of the genus *Micrococcus* resulted in the proposal of the genus *Nesterenkonia* (Stackebrandt *et al.*, 1995) and in the reclassification of *Micrococcus halobius* Onishi and Kamekura 1972 as *Nesterenkonia halobia* (Stackebrandt *et al.*, 1995). Recently, a second species of the genus *Nesterenkonia*, *Nesterenkonia lacusekhoensis*, has been proposed by Collins *et al.* (2002). In this work, we present the polyphasic taxonomic characterization of two halotolerant strains of the genus *Nesterenkonia* that were isolated from hypersaline soil samples from Xinjiang Province, western China.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains YIM 70084^T and YIM 70097^T are AY226508 and AY226510.

Images of cells of strains YIM 70084^T and YIM 70097^T are available as supplementary material in IJSEM Online.

Strains YIM 70084^T and YIM 70097^T were isolated using a modified glycerol/asparagine agar (ISP 5) medium (Shirling & Gottlieb, 1966) supplemented with 15 % (w/v) $MgCl_2.6H_2O$ and KCl, respectively. The isolation plates were incubated at 28 °C for 2 weeks. The purified strains were cultivated and maintained on medium containing 0·1 % (w/v) asparagine, 1 % glycerol, 0·1 % $K_2HPO_4.3H_2O$, 0·5 % yeast extract, 10 % $MgCl_2.6H_2O$ (for YIM 70084^T) or 10 % KCl (for YIM 70097^T). The pH was adjusted to 7·2 with 1 M NaOH. When required, the medium was solidified with 2 % (w/v) agar. Biomass for chemical and molecular systematic studies was grown in shaken flasks (~150 r.p.m.) at 28 °C for 1 week. Morphological properties were examined by light microscopy (Olympus microscope BH-2) and transmission electron microscopy with a Hitachi model H-800 TEM. Media and procedures used for determination of physiological features and carbon source utilization were those described by Shirling & Gottlieb (1966). The colony colour of strains grown on medium PYGV (Staley, 1968) and modified ISP 5 agar medium was determined by comparing the cultures with the most

suitable colour chips from the ISCC-NBS colour charts (Kelly, 1964).

Motility of cells was studied on LB swarming agar (0·3 %, w/v). The methods for measuring pH, temperature and salt tolerance were described by Tang *et al.* (2003). Some metabolic properties of the strains were determined by using API Coryne system with API ID 32 E test kits (bioMérieux) according to the manufacturer's instructions.

The sugars of purified cell walls were analysed as described by Stanek & Roberts (1974). Purified peptidoglycan preparations were obtained using the method described by Schleifer & Kandler (1972). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates (Merck) using the solvent systems of Schleifer & Kandler (1972). The amino-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985). Molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987). Analysis of enantiomers of peptidoglycan amino acids was performed by GC of *N*-pentafluoropropionyl amino acid isopropyl esters (Frank *et al.*, 1980) on an L-chirasil Val column (Macherey-Nagel) as described by Groth *et al.* (1997). Phospholipid analysis was carried out as described by Komagata & Suzuki (1987). Menaquinones were isolated using the method of Collins *et al.* (1977) and were analysed by HPLC (Groth *et al.*, 1997). Cellular fatty acid composition was performed as described by Sasser (1990) using the Microbial Identification System (MIDI Inc.).

DNA for base composition was prepared following the method of Marmur (1961) and the G+C content was determined using the thermal denaturation method of Marmur & Doty (1962) by using a UV-VIS spectrophotometer model UV1601 (Shimadzu). Extraction of genomic DNA and amplification of 16S rDNA were performed as described by Xu *et al.* (2003). Multiple alignments with sequences of a broad selection of *Actinobacteria* and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980, 1983). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Young cells (24–48 h) of YIM 70084^T were Gram-positive, non-spore-forming and motile cocci with flagella. Agar colonies were light orange–yellow to deep orange–yellow and their surface was smooth. The strain grew optimally in modified ISP 5 medium at 28 °C, at pH 7·0–8·0 and in the presence of 10·0 % (w/v) $MgCl_2.6H_2O$. Cells of strain YIM 70097^T were Gram-positive, non-motile, non-spore-forming, irregular rods. The colony colour was light yellow and the surface of colonies was smooth. It grew optimally in modified ISP 5 medium at 28 °C, at pH 8·0–9·0 and in the presence of 10·0 % KCl. Detailed physiological and biochemical characteristics of the two strains are given in Table 1 and in the species descriptions.

For strain YIM 70084^T, the peptidoglycan type was A4α (Schleifer & Kandler, 1972), with L-lysine in a tetrapeptide subunit and an interpeptide bridge consisting of Gly–Asp. Its cell-wall sugars were xylose and galactose and the menaquinone composition was MK-7, MK-8, MK-5, MK-6, MK-9 (ratio 80 : 11 : 5 : 1 : 0·5). Its polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified glycolipid. The cellular fatty acids were ai-$C_{15:0}$ (51·33 %), i-$C_{16:0}$ (17·32 %), ai-$C_{17:0}$ (13·74 %), ai-$C_{15:1}$ (9·97 %), $C_{14:0}$ (0·33 %), $C_{16:0}$ (3·26 %), i-$C_{14:0}$ (1·25 %), i-$C_{15:0}$ (1·12 %), ai-$C_{13:0}$ (0·35 %), i-$C_{15:1}$ (0·37 %), i-$C_{16:1}$ (0·49 %) and ai-$C_{17:1}\omega 9c$ (0·49 %). Strain YIM 70097^T differed from strain YIM 70084^T in displaying the interpeptide bridge Gly–L-Glu. Its cell-wall sugars were ribose and galactose and the menaquinone composition was MK-8, MK-7 and MK-9 (ratio 56 : 24 : 17). The phospholipid pattern contained diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine. The cellular fatty acids contained ai-$C_{15:0}$ (28·50 %), ai-$C_{17:0}$ (38·10 %), $C_{15:0}$ (0·26 %), $C_{16:0}$ (1·26 %), i-$C_{14:0}$ (0·31 %), i-$C_{15:0}$ (6·50 %), i-$C_{16:0}$ (8·97 %), i-$C_{17:0}$ (4·06 %), i-$C_{19:0}$ (0·26 %), i-$C_{15:1}$ (0·98 %), i-$C_{16:1}$ (3·48 %), ai-$C_{15:1}$ (0·43 %) and ai-$C_{17:1}\omega 9c$ (6·9 %). The DNA base compositions of strains YIM 70084^T and YIM 70097^T were 64·4 and 66·7 mol% G+C.

The lengths of the almost-complete 16S rDNA sequences analysed for strains YIM 70084^T and YIM 70097^T were 1490 and 1506 bp. The two isolates showed 96·9 % 16S rDNA sequence similarity to each other and represented a separate cluster within the genus *Nesterenkonia*. Whereas strain YIM 70097^T showed 96·8 % sequence similarity to both type strains of validly published *Nesterenkonia* species, the sequence similarities of strain YIM 70084^T to *N. halobia* DSM 20541^T and *N. lacusekhoensis* DSM 12544^T were respectively 96·5 and 96·3 %. A phylogenetic tree is shown in Fig. 1.

It is generally accepted that organisms displaying 16S rDNA sequence similarity values of 97 % or less do not belong to the same species (Stackebrandt & Goebel, 1994). It is therefore evident from the phylogenetic data and the great difference in phenotypic characteristics (Table 1) that the two isolates from hypersaline soils represent two previously unknown species. Thus, the novel species *Nesterenkonia halotolerans* sp. nov. (type strain YIM 70084^T) and *Nesterenkonia xinjiangensis* sp. nov. (type strain YIM 70097^T) are proposed.

Description of *Nesterenkonia halotolerans* sp. nov.

Nesterenkonia halotolerans (ha.lo.to′le.rans. Gr. n. *halos* salt; L. part. *tolerans* tolerating; N.L. pres. part. *halotolerans* referring to the ability to tolerate high salt concentrations).

Table 1. Comparison of phenotypic characteristics of strains YIM 70084^T and YIM 70097^T and species of genus *Nesterenkonia*

Data for reference species were taken from Collins *et al.* (2002). W, Weak reaction, ND, not determined. The G+C contents of strains YIM 70084^T and YIM 70097^T were determined by the thermal denaturation method; HPLC was used for the other strains.

Characteristic	YIM 70084^T	YIM 70097^T	*N. halobia* DSM 20541^T	*N. lacusekhoensis* DSM 12544^T
Morphology	Cocci	Short rods	Cocci in pairs or tetrads	Short rods, occasionally branching, and cocci
Colony pigmentation (PYGV medium)	Deep orange–yellow	Light yellow	Colourless	Bright yellow
Temperature range for growth (°C)	4–40	20–40	20–40	8·5 to >42
NaCl tolerance for growth (%)	0–25	0–25	5·0 to >23	0 to >15
pH tolerance	7·0–9·0	7·0–12·0	<6·0–10·0	7·5–9·5
Oxidase activity	−	−	+	−
Starch hydrolysis	−	−	+	−
H_2S production	−	−	−	W
Gelatin liquefaction	+	+	−	−
Carbon utilization:				
D-Fructose	+	+	ND	+
D-Mannose	+	+	−	+
Trehalose	−	−	−	+
Acid from:				
Mannitol	−	−	+	−
Xylose	−	−	+	−
Lactose	−	−	+	−
Trehalose	−	−	−	+
Galactose	−	−	W	−
Chemical characteristics:				
Peptidoglycan type	L-Lys–Gly–Asp	L-Lys–Gly–L-Glu	L-Lys–Gly–L-Glu	L-Lys–L-Glu
Polar lipids*	DPG, PG, PI, GL	DPG, PG, PC	DPG, PG, PI, GL	DPG, PG, PC, GL
Major menaquinones	MK-7, MK-8	MK-7, MK-8, MK-9	MK-7, MK-8, MK-9	MK-7, MK-8
Major cellular fatty acids (>10%)	ai-$C_{15:0}$ (51·3%); i-$C_{16:0}$ (17·3%); ai-$C_{17:0}$ (13·7%)	ai-$C_{17:0}$ (38·1%); ai-$C_{15:0}$ (28·5%)	ai-$C_{15:0}$ (65·0%); ai-$C_{17:0}$ (22·6%)	ai-$C_{15:0}$ (35·3%); i-$C_{16:0}$ (15·9%); i-$C_{15:0}$ (23·0%)
DNA G+C content (mol%)	64·4	66·7	71·5	66·1

*DPG, Diphosphatidylglycerol; GL, unknown glycolipid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

Cells are Gram-positive, non-spore-forming, motile cocci (see Supplementary Figure in IJSEM Online). The colony colour on modified ISP 5 medium is deep orange–yellow (the colour of the fringe) to light orange–yellow (the colour of the centre); some colonies resemble concentric rings. Colonies are circular, opaque and approximately 2·5–3·5 mm in diameter after 24 h at 28 °C. The optimum growth temperature is 28 °C. The optimum concentration of $MgCl_2.6H_2O$ is 10 %. The type strain is positive for gelatin liquefaction and urease production and negative for milk peptonization and coagulation, nitrate reduction, growth on cellulose, H_2S and melanin production and starch hydrolysis. The following substrates are utilized: glucose, galactose, mannose, fructose, sucrose, maltose, starch, lactose and dextrin. Ribose, arabinose, cellobiose, trehalose, sorbitol and xylose are not utilized. The peptidoglycan type is A4α, L-Lys–Gly–Asp. Cell-wall sugars are xylose and

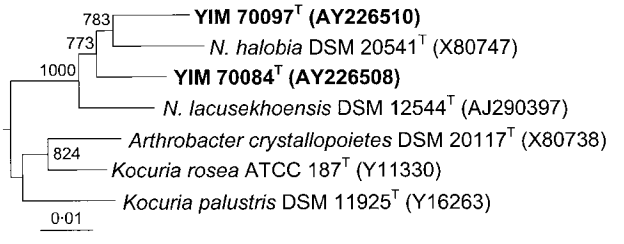

Fig. 1. Phylogenetic dendrogram obtained by distance-matrix analysis of 16S rDNA sequences, showing the position of strains YIM 70084^T and YIM 70097^T among phylogenetic neighbours. Numbers on branch nodes are bootstrap values (1000 resamplings). The sequence of *Streptomyces megasporus* DSM 41476^T (Z68100) was used as root. Bar, 1 % sequence divergence.

galactose. Main polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified glycolipid. Predominant menaquinones are MK-7 and MK-8. Major cellular fatty acids are ai-$C_{15:0}$, i-$C_{16:0}$ and ai-$C_{17:0}$. The DNA G+C content of the type strain is 64·4 mol%.

The type strain is YIM 70084^T (=CCTCC AA001022^T=DSM 15474^T), isolated from a saline soil sample from Xinjiang Province, China.

Description of *Nesterenkonia xinjiangensis* sp. nov.

Nesterenkonia xinjiangensis (xin.ji.ang.en′sis. N.L. fem. adj. *xinjiangensis* pertaining to Xinjiang, the province of western China in which the samples were collected).

Cells are Gram-positive, non-motile, non-spore-forming, diphtheroid, irregular rods (see Supplementary Figure in IJSEM Online). The colony colour on modified ISP 5 medium is light yellow. Colonies are circular, opaque, somewhat convex and approximately 3·5–4·5 mm in diameter after 24 h at 28 °C. The optimum growth temperature is 28 °C. The optimum concentration of KCl for growth is 10·0 %. The type strain is positive for gelatin liquefaction, milk peptonization and urease production and negative for milk coagulation, nitrate reduction, growth in cellulose, H_2S and melanin production and starch hydrolysis. Almost all tested carbon sources, including glucose, galactose, mannose, fructose, sucrose, maltose, starch, lactose, dextrin, ribose, arabinose, cellobiose and xylose, are utilized; trehalose and sorbitol are not utilized. The peptidoglycan type is A4α, L-Lys–Gly–L-Glu. The cell-wall sugars are ribose and galactose. Predominant menaquinones are MK-8, MK-7 and MK-9. Main phospholipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine. Major cellular fatty acids are ai-$C_{15:0}$ and ai-$C_{17:0}$. The DNA G+C content of the type strain is 66·7 mol%.

The type strain is YIM 70097^T (=CCTCC AA001025^T=DSM 15475^T), isolated from a saline soil sample from Xinjiang Province, China.

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